Effects of angiotensin-converting enzyme inhibition and calcium channel blockade on cardiac apoptosis in rats with 2K1C (two-kidney/one-clip) renovascular hypertension

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ABSTRACT

Apoptosis plays a role in the regulation of heart mass and architecture, and might contribute to the cardiac remodelling seen in renovascular hypertension. It is not known whether the beneficial effects of angiotensin-converting enzyme (ACE) inhibition or calcium channel blockade on cardiac remodelling are linked to the modulation of apoptosis. To test this hypothesis, we established four groups of rats: (i) sham-operated controls, (ii) a group that underwent the two-kidney/one-clip (2K1C) procedure, (iii) a group with 2K1C treated for 12 weeks with quinapril (6 mg·day\(^{-1}\)·kg\(^{-1}\)), and (iv) a group with 2K1C treated for 12 weeks with diltiazem (24 mg·day\(^{-1}\)·kg\(^{-1}\)). Treatment started 2 weeks after clipping. Systolic blood pressure was reduced to a similar extent by quinapril and diltiazem (2K1C, 223±19 mmHg; 2K1C + quinapril, 149±15 mmHg; 2K1C + diltiazem, 160±40 mmHg; both P < 0.01 compared with 2K1C alone). Left ventricular weight, interstitial fibrosis and perivascular fibrosis were reduced significantly by both drugs. The apoptotic index (apoptotic cells/total cell number) was increased 21.6-fold (P < 0.01) after quinapril treatment as compared with the 2K1C group, but was not affected by calcium channel blockade. In conclusion, our study demonstrates that ACE inhibition, in contrast with calcium channel blockade, may cause regression of cardiac hypertrophy/remodelling in 2K1C renovascular hypertensive rats through enhanced apoptosis.

INTRODUCTION

Hypertensive heart disease involves myocyte and non-myocyte growth that leads to adverse structural remodelling of the heart [1]. In hypertensive heart disease, it is not the quantity of myocardium, but rather the quality, that accounts for an increased risk of adverse cardiovascular events [1]. The effects of arterial hypertension on the myocardium occur in two distinct stages [2]. In both human patients and animal models, pressure overload is characterized by a period of compensation in which left ventricular concentric hypertrophy normalizes systolic wall stress, and contractile function is preserved. This period of adaptation, which...
may last for weeks in rodents and months to years in humans, is followed inexorably by a transition to cardiac failure. This transition is characterized by impaired survival, the onset of chamber dilatation with the failure of further concentric hypertrophic growth to normalize load, and progressive contractile dysfunction [3]. The structural homogeneity of cardiac tissue is governed by the existence of a balanced equilibrium between stimulatory and inhibitory signals that regulate cell growth, apoptosis, phenotype and matrix turnover. Stimulators (e.g. angiotensin II, aldosterone and endothelins) are normally counterbalanced by inhibitors (e.g. bradykinin, NO and prostaglandins) in a paradigm of reciprocal regulation. Cardioprotective agents such as angiotensin-converting enzyme (ACE) inhibitors counteract the imbalance between stimulators and inhibitors [4–6].

Apoptosis is an active, tightly regulated, energy-requiring process in which cell death follows a programmed sequence of events [7]. Fragmentation of chromosomal DNA is the biological hallmark of apoptosis [8]. This process of internucleosomal fragmentation of DNA appears to be a genetic event that requires gene transcription and translation [9], and may be stimulated or inhibited by a variety of regulatory factors, including growth factors and cytokines [10]. Apoptotic cells undergo extracellular degeneration or phagocytosis by macrophages and neighbouring cells [11]. A number of genes have been identified that regulate the apoptotic process. The Bcl-2 proto-oncogene family is critical for the regulation of apoptosis [12]. Bcl-2 family members come in two functional categories: those that inhibit apoptosis (e.g. Bcl-2) [13] and those that induce apoptosis (e.g. Bax) [14]. The relative abundance of pro-apoptotic and anti-apoptotic proteins determines susceptibility to cell death [15]. Thus it has been proposed that cell viability after an apoptotic stimulus may depend on the ratio of the level of Bcl-2 to that of Bax [16]. A high level of Bcl-2 relative to Bax promotes survival, whereas an excess of Bax relative to Bcl-2 promotes death [14].

The regulation of apoptosis is altered in cardiac cells of spontaneously hypertensive rats (SHRs), and the local renin–angiotensin system may be involved in such an alteration [17]. Diez et al. [17] found evidence suggesting increased susceptibility to apoptosis of coronary smooth muscle cells from SHRs that had received long-term treatment with an ACE inhibitor [17]. Tea et al. [18] found that the ACE inhibitor enalapril stimulated DNA fragmentation in SHRs at 2 and 4 weeks. Thus the ACE-inhibitor-induced regression of cardiac hypertrophy was associated with increased apoptotic activity.

In the present study, we analysed whether apoptosis is involved in the process of left ventricular remodelling in rats with renovascular hypertension induced by the two-kidney/one-clip (2K1C) procedure. In particular, we studied whether the ACE inhibitor quinapril or the calcium channel blocker diltiazem affect the rate of apoptosis as part of the mechanisms involved in the regression of cardiac remodelling.

**MATERIALS AND METHODS**

**Materials**

The ACE inhibitor quinapril and the calcium channel blocker diltiazem hydrochloride were generously donated by Gödecke AG. The standard rat breeding food Altromin® was obtained from Altromin GmbH. Unless otherwise stated, all reagents were of analytical grade and were purchased from Merck, Boehringer-Mannheim or Sigma.

**Animal experiments**

Male Wistar–Kyoto rats were obtained from Møllegard (Schönwalde, Germany), and were allowed free access to food and water. Renovascular hypertension was induced by the Goldblatt 2K1C method adapted to the rat [19]. A silver clip (internal diameter 0.2 mm) was placed over the left renal artery of 9-week-old male Wistar–Kyoto rats. Anaesthesia was performed with an intraperitoneal injection of a mixture containing ketamine (80 mg/kg) and rompun (12 mg/kg). The animal experiment was conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Four groups were established: (1) sham-operated rats (n = 14); (2) rats with renovascular hypertension (2K1C) (n = 6); (3) rats with renovascular hypertension (2K1C) treated with quinapril (n = 7); and (4) rats with renovascular hypertension (2K1C) treated with diltiazem (n = 8).

The rats were clipped on day 1 of week 0, drug treatment was started on day 7 of week 2, and the animals were killed after 12 weeks of treatment (14th week of the experiment). Blood pressure was measured at 1-week intervals during the first 4 weeks, and then every 3 weeks until the end of the experiment. Drugs were administered in the drinking water. The rats received a daily dose of 6 mg/kg quinapril or 24.5 mg/kg diltiazem. Water uptake and body weight were measured repeatedly in order to adjust the concentrations of inhibitors to allow maintenance of a constant dose. The animals tolerated the treatment very well. None of the animals died, and thus all rats could be analysed. We chose a treatment period of 3 months because left ventricular hypertrophy and fibrosis is clearly detectable at that time point. At earlier time points these cardiac alterations are less pronounced.

**Measurement of blood pressure**

Measurement of arterial blood pressure was performed as described by Hocher et al. [20,21]. The rats were placed into a retaining box and warmed for 15 min, after which time the pulse became detectable at the tail using a piezo element. Systolic pressure was measured using a tail cuff and a pressure transducer in conjunction with an auto-
matic pressure delivery system and chart recorder (Harvard indirect rat tail blood pressure system; Harvard Apparatus Ltd, Edenbridge, Kent, U.K.). Blood pressure values obtained using this indirect measurement technique are in good agreement with values from direct arterial blood pressure measurements. However, it is important to note that warming up the animals led to a minor decrease in blood pressure. This effect was similar in all groups.

**Histological evaluation**

For pathohistological evaluation, all samples were embedded in paraffin, cut into 3 μm sections, submitted to haematoxylin/eosin or Sirius Red staining and analysed as described previously [22–25]. The samples were always obtained from the middle part of the left ventricle, in order to exclude possible differences in matrix protein content in different regions of the left ventricle. The media/lumen ratio of the intracardiac arteries was analysed using a video microscope connected to a PC using the Image 1.61 program. For calculation of the media/lumen ratio, we measured the areas of both the media and the lumen.

The severity of cardiac fibrosis was evaluated after Sirius Red staining. We measured the relationship of the red-stained area (connective tissue) to the total area of the whole heart section. The data thus obtained were assessed using a video microscope connected to a PC using the Image 1.61 program. Perivascular fibrosis of intracardiac arteries was assessed in 25 randomly selected arteries per sample observed at 400 × magnification using the following scale: 0, no perivascular fibrosis; 1, minor perivascular fibrosis; 2, moderate perivascular fibrosis; 3, marked perivascular fibrosis and 4, very marked perivascular fibrosis. All tissue samples for scoring were evaluated independently by two investigators without prior knowledge of the group to which the rats belonged.

**Detection of apoptotic cells by the TUNEL method**

A standard in situ terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method was used to identify apoptotic cells within heart tissue sections. TUNEL was performed according to the method of Gavrieli et al. [26], with some modifications. Tissue samples were fixed in Bouin’s fixant (2.5% copper acetate/4% picric acid in 3.5% formaldehyde in distilled water), and embedded in paraffin. For a positive control, TUNEL was performed after DNase treatment. For a negative control, TUNEL was performed without the addition of deoxyribonucleotidyl transferase. Briefly, 3 μm-thick paraffin sections were de-waxed, incubated with 30 μg/ml proteinase K for 30 min at room temperature, washed, and then endogenous peroxidase was inactivated by covering the sections with 2% H₂O₂ for 5 min at room temperature. The sections were then washed and treated with 0.1% Triton X-100 in 0.1% sodium citrate for 3 min at room temperature, washed again and then labelled with deoxyribonucleotidyl transferase (1:200) and biotin-dUTP labelling mixture (1:100) in deoxyribonucleotidyl transferase reaction buffer for 60 min at 37°C. The reaction was terminated by immersing the slides in TB buffer (0.1 mol/l Tris, 0.05 mol/l MgCl₂, pH 7.5) at room temperature for 15 min. After washing in PBS for 5 min at room temperature, sections were blocked with 2% (w/v) BSA for 10 min at room temperature, incubated with avidin-biotinylated horseradish peroxidase for 10 min at 37°C, washed with PBS and then developed with 3’-amino-9-ethylcarbazole solution to give a brown product. We counted TUNEL-positive cells and the total cell number in the left ventricle of the heart, in order to calculate an apoptotic index (TUNEL-positive cells/total cell number).

**Nuclear staining with propidium iodide**

As described by Paul et al. [27], tissue samples were fixed in formalin and embedded in paraffin. Sections of 3 μm thickness were de-waxed with xylene for 2 × 10 min. After dehydration from 96% to 70% (v/v) ethanol for 5 min, tissue sections were washed with PBS for 5 min. All samples were incubated with propidium iodide solution (4 mg propidium iodide/l) containing RNase A (100 mg/l) for 10 min. After washing with PBS, all slides were covered with glycerin/PBS (1:2, v/v). Apoptotic cells were counted with a confocal microscope at 400 × magnification.

**Immunohistochemical determination of Bax and Bcl-2**

Immunohistochemistry was performed according to the method of Krajewski et al. [28], with some modifications. Paraffin-embedded tissue sections (5 μm) were de-waxed. After dehydration for 15 s, slides were incubated in 2% (v/v) H₂O₂ in methanol for 30–45 s, washed with 90% (v/v) ethanol for 20 s, followed sequentially by 70% ethanol for 20 s, distilled water for 1 min and PBS (120 mmol/l NaCl, 11.5 mmol/l NaH₂PO₄, 31.3 mmol/l KH₂PO₄, pH 7.4–7.6) for 5 min, and then heated briefly in a microwave in acidic buffer (10 mol/l Tris, pH 6) for a total of 5.5 min essentially as described, and washed in PBS for 2 × 5 min. Tissue sections were pre-blocked for 30–45 min in TNK buffer (100 mol/l Tris, pH 7.6–7.8; 550 mmol/l NaCl, 10 mmol/l KCl) containing 2% (w/v) BSA, 0.1% Triton X-100 and 1% (v/v) normal goat serum. Pre-immune, anti-Bax (1:1000–1:2000) or anti-Bcl-2 (1:800–1:1500) serum was added to the slides in the same solution and incubated overnight at room temperature on a shaker. After washing with PBS, tissue sections were incubated for 1 h with 2.8 μg/ml
biotinylated goat anti-(rabbit IgG) antibody in TNK buffer containing 0.5% normal mouse serum, and then washed and incubated for 50 min with a streptavidin–alkaline phosphatase complex reagent in the same buffer (1:500). After washing with PBS, colour development was achieved by incubation for 20 min with a solution containing Fast Red in substrate buffer (0.2 mg/ml levamisol, 0.25 mg/ml naphthol, 0.1 mol/l Tris/HCl, pH 8.2) Counterstaining was with haematoxylin.

Analysis of data
After testing for heterogeneity of variance, the unpaired Student’s \( t \) test was used to determine statistical differences between group means for cardiac weight, computer-aided analysis of the media/lumen ratio of cardiac blood vessels, cardiac apoptosis and cardiac fibrosis. The Mann–Whitney \( U \) test was used for analysis of statistical differences after scoring of perivascular fibrosis. Mean arterial blood pressure values during the time on medication were compared using ANOVA for repeated measurements and tested with Bonferroni’s method for significant differences. Results were considered significantly different at \( P < 0.05 \).

RESULTS

Effects of quinapril and diltiazem on blood pressure, heart weight, cardiac fibrosis and vascular remodelling in rats with 2K1C renovascular hypertension

The dosages of quinapril and diltiazem used in the present study reduced blood pressure to a similar extent in rats with renovascular hypertension. Heart rate was not affected during therapy with either drug (Table 1 and Figure 1). Blood pressure was relatively low in all groups, especially in the sham group. This is most probably due to the method used for blood pressure detection: the rats were warmed up in order to detect a pulse in the tail. This was done in a similar way for all groups.

Body weight was lower in the hypertensive animals compared with sham-operated rats. Heart weight was reduced significantly by both drugs (\( P < 0.05 \) compared with untreated 2K1C rats; \( * P < 0.05 \) compared with untreated 2K1C rats. MAP, mean arterial pressure.

![Figure 1](image)

**Figure 1** Systolic blood pressure in rats with renovascular hypertension treated with quinapril or diltiazem in comparison with untreated rats with renovascular hypertension and sham-operated controls

Treatment was started 1 week after clipping. Quinapril and diltiazem reduced blood pressure in 2K1C renovascular hypertensive rats to a similar extent. Data are given as means ± S.E.M.; \( * P < 0.05 \) compared with untreated 2K1C rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham-operated</th>
<th>2K1C</th>
<th>2K1C + quinapril</th>
<th>2K1C + diltiazem</th>
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<td>Bax (Bax-positive cells/10000 cells)</td>
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<td>Media/lumen ratio</td>
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<td>Cardiac cell density (cell nuclei/ 100000 ( \mu )m(^2))</td>
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Values were measured 3 months after clipping. Data are given as means ± S.D. (\( n = 6–8 \) for each group); \( * P < 0.05 \) compared with untreated 2K1C rats; \( † P < 0.05 \) compared with sham-operated rats.
and perivascular fibrosis of intracardiac arteries were markedly reduced by treatment with either quinapril or diltiazem (Figure 2).

**Effects of ACE inhibition and calcium channel blockade on cardiac apoptosis**

The occurrence of apoptosis within the hearts of rats with renovascular hypertension was analysed by two independent methods: the TUNEL method, and after nuclear staining with propidium iodide. Renovascular hypertension on its own did not induce an increased rate of cardiac apoptosis (Figure 3). The ACE inhibitor quinapril, on the other hand, induced a huge (21.6-fold) increase in the number of apoptotic cells within the left ventricle of rats with renovascular hypertension. The ACE-inhibitor-induced apoptosis was located within the myocardium and preferentially around intracardiac blood vessels (Figure 3). The rate of apoptosis induced by quinapril was in good agreement with a reduced cardiac cell density in the quinapril-treated 2K1C group (Table 1). Diltiazem treatment led to similar decreases in blood pressure (Figure 1), heart weight (Table 1), and perivascular (Figure 2A) and interstitial (Figure 2B) fibrosis, but had no effect at all on the rate of cardiac apoptosis (Figure 3) or cell density (Table 1).

The expression of Bax and Bcl-2, two major apoptosis-controlling proteins, was not altered by treatment with quinapril or diltiazem, indicating that ACE inhibition...
DISCUSSION

In the present study, we demonstrate that the remodelling process in the non-failing heart in rats with 2K1C renovascular hypertension is not associated with an increased rate of apoptosis. However, the beneficial effect of ACE inhibition on cardiac remodelling in this model is associated with a markedly increased rate of apoptosis. This effect was specific to ACE inhibition, because calcium channel blockade, which lowered blood pressure to a similar extent, had no effect on cardiac apoptosis.

Apoptosis maintains tissue integrity by counterbalancing cellular proliferation [29]. Apoptosis has been clearly demonstrated to be involved in cardiac remodelling under conditions of heart failure. In the failing heart, apoptosis seems to contribute to the loss of cardiomyocytes, whereas cardiac apoptosis is not enhanced in the non-failing heart. This has been shown in animal models of heart failure, and also in humans [3,7,30]. Our present data showing no increase in the rate of apoptosis in non-failing hearts of rats with renovascular hypertension are in good agreement with this concept. Others have reported similar findings in SHRs [30]. SHRs characterized by compensated left ventricular hypertrophy, normal fractional shortening and normal end-systolic wall stress had a normal cardiac apoptosis rate. In contrast, hearts from older SHRs with decompenated dilatation [30], characterized by a decrease in fractional shortening and an increase in end-systolic left ventricular pressure, exhibited a greatly increased rate of cardiac apoptosis.

Our present study shows for the first time that the beneficial effects of ACE inhibition in the non-failing hearts of rats with renovascular hypertension are linked specifically to apoptosis. This is in agreement with cell culture experiments showing that the induction of apoptosis may be a mechanism by which ACE inhibitors affect cardiovascular remodelling [31]. In addition, Tea et al. [18] demonstrated a potential role for apoptosis in cardiovascular therapy in 10–12-week-old SHRs without established heart failure. They treated the rats for 1–4 weeks with enalapril, propranolol, losartan, nifedipine, hydralazine or hydrochlorothiazide. Transient induction of apoptosis in the subepicardium appears to be a characteristic feature of the early response to the drug-induced regression of cardiac hypertrophy in the SHR.

Based on our present data and on previous reports, we suggest that blockade of the renin–angiotensin system induces apoptosis in the non-failing heart, leading to a lower cardiac cell density. This pharmacologically induced apoptosis of cardiac cells seems to play an important role in the beneficial effects of ACE inhibition on left ventricular hypertrophy in the non-failing heart. The molecular mechanism behind the induction of apoptosis by quinapril in 2K1C rats is unknown at present. Our data exclude a major role for Bax and Bcl-2 in this process. Bcl-2 family members consist of two functional categories: those that inhibit apoptosis (Bcl-2, Bcl-xL) and those that enhance apoptosis (Bax). The determining factor is the ratio of the levels of death-inhibiting Bcl-2 family members relative to those of death-promoting Bcl-2 family members [14]. Ikeda et al. [30] found increased expression of Bcl-xL at the failing stage in the SHR model, whereas the expression of Bax and Bcl-2 was unchanged, as in the present study.

In this context, it is important to note that mast cells cause apoptosis of cardiomyocytes in vitro [32] and in vivo [33]. This was demonstrated in 36-week-old SHRs with established left ventricular hypertrophy. However, further studies are needed to elucidate the molecular mechanism behind the induction of apoptosis by ACE inhibition (e.g. activation of mast cell-induced apoptosis of cardiomyocytes) in the non-failing heart in rats with renovascular hypertension.

In conclusion, our study has revealed that apoptosis on its own does not play a major role in the process of cardiac remodelling in 2K1C renovascular hypertensive rats, whereas blockade of the renin–angiotensin system in this model is linked specifically to a markedly increased (21.6-fold) rate of apoptosis, and to decreased intracardial and perivascular fibrosis, in a blood pressure-independent manner. We suggest that ACE inhibition induces a beneficial apoptotic process under conditions of left.
ventricular hypertrophy, at least in rats with renovascular hypertension.

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